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## Annual Report for Grant DAMD17-94-J-4203

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Annual Report: Grant DAMD17-94-J-4203  
September 1, 1997 - August 31, 1998

**Principal Investigator: Peter Gann, M.D., Sc.D.**

## INTRODUCTION

The overall aim of this project is to develop new biological markers that can improve epidemiological investigations into the etiology of breast cancer. Our studies encompass three types of novel biomarkers: a) *breast fluid from nipple aspirates for measurement of growth factors and steroids*, b) *saliva for measurement of sex steroid concentrations*, and c) *normal breast tissue from biopsy samples for assessment of lobular differentiation*. These biomarkers will allow epidemiologists to study the development of breast cancer in greater biological detail than previously possible using conventional questionnaire-based research and will also allow clinical investigators to test the effects of dietary or chemopreventive interventions. **To allow consistency with our previous reports, we will refer to all activities related to hormone or growth factor levels as Project 1; activities related to lobular differentiation will be referred to as Project 2.**

In breast fluid, we have been studying concentrations of growth factors, including epidermal growth factor (EGF), TGF- $\alpha$  and TGF- $\beta$ , that are presumed to play a major role in controlling breast cell proliferation and differentiation. We seek to determine what extraneous factors influence these GF levels in breast fluid, whether GF levels are associated with breast cancer, and ultimately, whether GF levels are modulated by pro- or anti-carcinogenic exposures. We have completed an evaluation of assay sensitivity and precision for EGF and TGF- $\alpha$ , ascertained the relative amounts of intra- versus inter-woman variability, and evaluated the association between plasma hormones, menstrual cycle position and GF levels. A manuscript describing these studies was published in *Cancer Epidemiology, Biomarkers & Prevention* (Gann P, et al., vol 6:421-428, 1997). Having established that the assay was sensitive and reliable, and that women secrete consistent yet individually distinct amounts of EGF and TGF- $\alpha$  in breast fluid, we next addressed the hypothesis that levels of these mitogenic growth factors are correlated with breast tissue density as determined by mammograms. Mammographic density is presently accepted as an independent risk factor for breast cancer. Assays for growth factors in women with mammographic density measurements are currently underway. During the past year, we also have focused attention on development of the assay for estradiol in breast fluid and have also conducted preliminary evaluations for TGF- $\beta$ 1 and IGF-1.

Our work with saliva samples focuses on development of new assay methods for estradiol and progesterone. These assays are direct assays - they avoid an extraction step that requires a large volume of sample and can introduce error. The new assays will provide ultrasensitive non-invasive methods for serial measurement of steroid hormone concentrations in premenopausal women. During the past year, we have completed validation work on both assays. A revised manuscript describing the assays is currently under review; a second manuscript concerning the 'tracking' of salivary steroid levels *between* menstrual cycles in individual women is currently in preparation.

Project 2 deals with measurement of lobular differentiation in normal breast tissue. We postulate that it is feasible to use normal breast tissue from the margins of breast biopsies to obtain a histological index of the differentiation status of a woman's breast. This idea follows from the work of Russo, et al., which demonstrates the feasibility and usefulness of such a marker in a rodent model. Data on human lobular differentiation previously has been obtained from breast reduction or autopsy specimens, and therefore are not abundant. Development of a histologic differentiation marker that can be used in readily available tissue would allow us to begin epidemiological studies aimed at identifying the major influences on human breast differentiation. It is hypothesized that extensive lobular differentiation will protect against breast cancer development.

We have previously demonstrated acceptable inter-reader reliability for a method involving categorization of lobules into 3 types according to the number of branching acini. We have also observed homogeneity in lobule type across regions of the same breast. We have now nearly completed analysis of a small case-control study comparing lobule type between women with breast cancer and women with benign breast disease.

## **BODY OF THE REPORT**

### **A. Methods and Procedures: Project 1 (Hormones/Growth Factor Levels in Body Fluids, and Their Determinants)**

#### **1. Laboratory assays for breast fluid growth factors**

Our efforts primarily focused on IGF-1. Circulating levels of IGF-1 recently have been associated with an increased risk of breast and prostate cancer in prospective studies. We have conducted assay development on both breast fluid and expressed prostatic fluid (EPF) in parallel because of the similar epidemiologic findings and because prostatic fluid is more abundant and more easily obtained. Original attempts at measuring IGF-1 in prostatic and breast fluid were made with the DSL coated tube IRMA kits (both with and without extraction). A range of dilutions was assayed, from undiluted to 1:100 in EPF, and 1:5 to 1:40 in breast fluid. An unusual trend appeared in that as the dilutions became higher, so did the measured values of IGF-1. It was thought that there was some type of inhibitor present that might be lost with increasing dilution. A larger range of dilutions was attempted for EPF, from 1:50 to 1:1600. Consistent results from these dilutions could not be reproduced. We then chose to work with the Nichols Diagnostics radioisotopic assay kit, with extraction. The kit suggests two methods for extraction. The first method we attempted was a C18 Sep-Pak column extraction. While detectable levels were found in serum, we did not find detectable levels in EPF or breast fluid. In addition, we had complications with the soft pellet produced during the assay. We examined both concentrated EPF samples, as well as fresh, flash frozen EPF samples, and detected very small amounts of IGF-1. After numerous attempts to correct for the soft pellet, we changed to the acid-ethanol extraction procedure, which produced a more solid pellet. Exploratory assays on both concentrated and flash frozen breast fluid and EPF samples are underway.

#### **2. Laboratory assays for breast fluid estrogens**

Original attempts at measuring estradiol were made with DELFIA, a time-resolved fluoroimmunoassay produced by Wallac Oy, Inc (Turku, Finland). A considerable amount of background measurement was present that we could not eliminate. We then chose to work with the ultrasensitive radioimmunoassay produced by Diagnostic Systems

Laboratories (DSL, Webster, TX). With modification of this kit, we were able to measure detectable levels of estradiol at dilutions as great as 1:400.

In order to confirm that what we were measuring is actually estradiol, we have attempted a number of extraction and purification procedures. Our first attempt involved the use of ether to extract the steroid. The sample was extracted three times with three volumes of ether. The ether layers were dried under nitrogen in a warm water bath, reconstituted with PBS with 0.1% gelatin, pH 7.0, and assayed with the DSL kit. The measurements seen with these samples were unusually high (80-90 pg/mL). These values were much higher than what we had seen when assaying the unextracted breast fluid. In addition, when the aqueous residue remaining after ether extraction was assayed, it often produced a value as high as non-extracted breast fluid. It appeared that the ether did not remove the estradiol, and also interfered with the assay. An alternative solvent, ethyl acetate, is currently being used. Both ether and ethyl acetate have been tested simultaneously for their background effects. While the measurement produced by ethyl acetate is negligible, the value produced by ether was around 7 pg/mL.

To test the extraction efficiency of the ethyl acetate, a number of samples spiked with increasing amounts of estradiol were extracted twice with three volumes of ethyl acetate. The extracts were dried under nitrogen in a warm water bath and assayed using the DSL kit. The results were variable, with recovery rates ranging from 63-96%.

We are currently examining a system of three purification techniques: 1. an ethyl acetate extraction, 2. a partition between 0.4N NaOH and isooctane for separation of phenolic from neutral steroids, and 3. a Sephadex LH-20 column separation. Each one produces a slightly more complete purification. By comparing the results from these three steps, we will be able to see the extent of purification needed. The procedure, in brief, is as follows: Aliquots of PBS-gelatin are spiked with increasing amounts of estradiol. The samples are extracted with ethyl acetate in the same manner mentioned above. A small aliquot is taken out for assay after this step, and the remaining sample is dried down and reconstituted with isooctane. It then proceeds through the phenolic extraction, where it is extracted twice with an equal amount of 0.4M NaOH. The two extracts are combined and neutralized with an equal volume of 0.4M HCl. The entire volume is then extracted twice with ethyl acetate. Another small aliquot is taken out for assay after this step, and the remaining extract is dried down and reconstituted with toluene:methanol 85:15. It is then transferred to a column constructed with Sephadex LH-20, and eluted with the same solvent mixture. The chromatographic behavior of estradiol on the column was tested separately using tritiated estradiol. Based on that information, the 12<sup>th</sup> through the 21<sup>st</sup> milliliters from the column are collected. All samples are assayed in PBS-gelatin using the DSL radioimmunoassay.

PBS-gelatin, spiked with tritiated estradiol, was run through all three steps. Recovery for the three purification steps was 81%, 77%, and 62% respectively. The problem we are currently having is with the blank that we run through the above procedure. The values received for this sample have been unexpectedly high (2-10 pg/mL). We are currently examining this problem and measuring the estradiol by RIA at each purification step.

### **3. Mammographic Density Study**

The Mammographic Density Study (MDS) was begun in 1996 with the following aims: 1) to evaluate the association between breast fluid levels of EGF and TGF- $\alpha$  and breast parenchymal density as reflected in screening mammograms, and 2) to evaluate the



association between these breast fluid GF levels and reproductive risk factors for breast cancer. Aim 2 corresponds to Study 4 (Task 4 in SOW) in our original USAMRDC proposal. Mammographic density is increasingly recognized as a risk factor for breast cancer development and appears to be controlled, at least partially, by ovarian hormonal influences. We hypothesize that women with characteristically high levels of estradiol and mitogenic growth factors in breast fluid will have increased mammographic density.

Following IRB approval, we began to collect breast fluid from women receiving mammograms at the Lynn Sage Breast Center at Northwestern. Women were eligible if they were between 35-60 years old, had no history of breast cancer, were at least 2 years post-lactation, were scheduled for a screening mammogram as opposed to a mammogram for follow-up of an abnormal finding, and had no nipple soreness or lesions that would preclude breast fluid sampling. After the films were taken, radiology technicians gave each eligible woman a cover letter and consent form to review and sign while waiting (still in examination gown) for the film quality to be checked. Thus, all nipple aspirations were performed soon after breast compression. Following nipple aspiration, an interviewer administered a brief questionnaire to obtain supplemental information on reproductive history, exogenous hormone use and family history of breast cancer. A staff member validated according to procedures described earlier in the project traced the whole breast area and areas of density on the cranio-caudal view mammogram. These tracings were then re-traced with a computerized planimeter to measure total breast area and total area occupied by radiographic densities.

Breast fluid EGF measurements were completed for this study during the previous project year. During the project year immediately past, we have performed assays for TGF- $\alpha$  in breast fluid samples from MDS participants. The details of the TGF- $\alpha$  assay are described in our previous publications.

#### **4. The association between breast fluid growth factor levels and reproductive/lifestyle factors**

We have accumulated enough data on EGF and TGF- $\alpha$  levels in women from various studies to permit exploratory analyses examining possible determinants of these levels. We have combined data from the Repeat Sample Study (RSS – described in previous reports) and the MDS for these analyses. We have data on factors pertaining to lifestyle and reproductive history for each of these women, including smoking history, height, body mass index, use of oral contraceptives or estrogen replacement, alcohol consumption, menarche, menopausal status, number and timing of pregnancies, and lactation. Average values for breast fluid EGF for each individual are log-transformed to normalize the distribution for parametric analyses. Using SAS-PC, we are examining the association between lnEGF and the above variables.

#### **5. Assays for saliva progesterone and estradiol**

To measure progesterone in saliva directly without extraction, we use an ultrasensitive competitive-binding radioimmunoassay (RIA). The antibodies for the assay were produced by Dr. Robert Chatterton, whose laboratory conducts the assay. In the assay, 200  $\mu$ l samples of saliva are mixed with radiolabelled progesterone and progesterone antibody. The resulting bound progesterone is separated using dextran-coated charcoal, counted in a scintillation counter, and counts are compared to a standard curve. For salivary estradiol, we previously evaluated a time-resolved fluoroimmunoassay (DELFI), but have now switched to an ultrasensitive RIA (DSL, Webster, TX) that we have adapted and optimized for this



project. In this assay,  $^{125}\text{I}$ -labeled estradiol competes with salivary estradiol for binding to antibody in buffer solution. Addition of anti-IgG results in the formation of antigen-antibody complexes, which are then precipitated and separated from unbound hormone by centrifugation. This assay thus avoids potential problems caused by interference between substances in saliva and binding to the solid-phase secondary antibody used in DELFIA.

Optimization and validation of the assay for salivary estradiol was an arduous task, given our goal of measuring the low concentrations present in saliva without an extraction step. The RIA kit is optimized for measurement of estradiol in serum. We experimented with numerous antigen:antibody ratios and sample volumes to construct a standard curve that is linear and steep within the expected range of sample results. We measured both intra- and interassay variability in a variety of quality control pool samples. We conducted serial dilution experiments comparing observed to expected estradiol values. We also added known amounts of estradiol to charcoal-stripped male saliva samples to compare observed to expected values. Our final procedure calls for 400  $\mu\text{l}$  of saliva for each assay.

#### **6. The Repeat Measures Study (RMS), the Conception Study and The Two-Cycle Preliminary Study (TCPS)**

We have tested our salivary hormone assays in three independent populations of volunteers. To address earlier problems with low serum-saliva correlation that we encountered with our initial assay methods, we developed the RMS to determine if correlations would be improved by looking at multiple serum-saliva pairs from the same woman. Informed consent was obtained from 9 premenopausal volunteers, who then provided fasting blood samples and both morning and evening saliva samples on the same day, for 2-3 days per week over an entire menstrual cycle. Salivary estradiol was measured using our recently-optimized assay (see above) and in serum by standard RIA. Assays were completed for 7 individuals, with each individual's samples assayed in a single run to avoid interassay variability. Scatterplots and correlations were produced for each individual.

To determine whether daily profiles for salivary progesterone and estradiol corresponded to expected (i.e., serum) patterns across the menstrual cycle, we measured levels in daily samples from 14 normal volunteers – aged 23-39 – who were planning a pregnancy (the Conception Study). Eleven of these women provided complete sets of saliva for each day of both a non-conception and a conception cycle.

The TCPS – to review briefly – is a group of 20 normal-cycling, non-conceiving women who were studied in our Clinical Research Center. These women provided daily saliva samples over two consecutive menstrual cycles, plus serum samples timed at 7 days after the LH peak in urine. One of the largest efforts over the previous year was the measurement of both estradiol and progesterone in these samples – a total of nearly 800 progesterone assays and 1000 estradiol assays. These data permitted us to evaluate several important issues involved in applying salivary steroid measurements to clinical or epidemiological research, including the consistency of steroid levels between cycles in individual women, relative to the amount of variation between women. Since it is impractical and costly to assay each daily sample separately in application studies, we also examined the effects on variance of combining daily saliva measurements in various ways.

## **B. Results/Discussion: Project 1**

### **1. Breast fluid growth factors**

The results achieved for IGF-1 are included above under Methods. We are not yet confident enough in the assay to report results for individual breast fluid samples.

### **2. Breast fluid estradiol**

These results, which also pertain to obstacles encountered during basic assay development, are described under Methods. Our plan remains, once the assay protocol is finalized, to test for within vs. between person variability, correlation between right and left breasts, and the association between synchronous serum and breast fluid estradiol concentrations.

### **3. Mammographic Density Study**

The TGF- $\alpha$  assay was run on breast fluid samples from the Mammographic Density Study using the radioimmunoassay from Biomedical Technologies (Stoughton, MA). As a part of an amended protocol, the samples were centrifuged and only the supernatant was used. Since the sample sizes were small, it was difficult to pipette the supernatant, and this had an effect on how many samples could be assayed. Most of the measured values came out below the value of the lowest standard on the standard curve. After some discussion, it has been determined that the amount of lipid in the breast fluid sample is negligible, and that a centrifugation step is not necessary. There are still sufficient sample volumes remaining from this study, and another run is currently being planned.

### **4. The association between breast fluid growth factor levels and reproductive/lifestyle factors**

Results thus far indicate interesting associations between breast fluid EGF concentration and age, BMI, age at menarche, oral contraceptive use and smoking history. Women under age 35 had significantly lower EGF levels than women aged 35 and older. BMI was positively associated with breast fluid EGF, with borderline statistical significance. Eight women had menarche at less than age 12, and their EGF levels were significantly lower than those for women with older ages at menarche. Women who had ever used oral contraceptives had EGF levels almost 2-fold higher than those never using them. However, the strongest association observed was for smoking history. Past or current smokers had a mean EGF level of 781 ng/ml, compared to 324 ng/ml among never smokers ( $P = 0.009$ ).

We are continuing the analysis of these data in order to address confounding and clarify independent associations. We plan to perform similar analyses for TGF- $\alpha$  once data from the MDS are available.

### **5. Salivary progesterone and estradiol**

We have obtained numerous results supporting the reliability and validity of the new salivary estradiol assay. In samples from male volunteers, the assay was able to discriminate differences in added estradiol as small as 2 pmol/L. Dilution studies over a 64-fold range gave observed values that were on average 106% (s.d. 6.2) of expected values. The results of experiments involving recovery of estradiol added to stripped samples are shown in Table 1 below. Observed values were again 106% (s.d. 4.3) of those predicted.

Table 1: Recovery of added estradiol (pmol/L) to pools of saliva with different endogenous concentrations of E<sub>2</sub>.

Sample	Endogenous	Added	Expected	Observed	Recovery
I	7.0*	5.7	12.7	12.0	94%
		11.5	18.5	19.2	104
		23.0	30.0	36.4	121
		45.9	52.9	53.1	100
II	13.4	11.5	24.9	24.5	98
		23.0	36.4	38.0	104
		45.9	59.3	62.2	105
III	26.7	11.5	38.2	40.6	106
		23.0	49.7	51.8	104
		45.9	72.6	83.9	116
IV	41.4	11.5	52.9	61.4	116
		23.0	64.3	63.0	98
		45.9	87.3	97.6	112

\* Male saliva pool. Other pools are from female subjects

We have also achieved good results for the correlation between serum and saliva, as shown for 7 subjects in the RMS in Table 2 below.

Table 2: Correlation of saliva and serum estradiol: repeated sampling from the same women within a single menstrual cycle.

Subject	Number of saliva/serum pairs	Pearson r	P value
1	7	- 0.42	- 0.34
2	12	0.71	0.01
3	10	0.85	0.002
4	8	0.84	0.01
5	10	0.40	0.25
6	12	0.63	0.03
7	12	0.71	0.01
All	71	- 0.08	0.51
Excluding Subject 1	64	0.21	0.09

Correlations for 6 of the subjects ranged between 0.40 and 0.85, with a median correlation of 0.71 overall. Serum-saliva correlations were statistically significantly different from 0 for 5 subjects despite the small number of datapoints per subject. The one outlier (subject 1) had the fewest data pairs and had 2 unexplained outlier saliva values that were more than 3 s.d. from the group mean. We suspected blood contamination in the sample (which can occur, for example, if the subject brushes her teeth vigorously before collecting saliva), but blood was not visible and guaiac tests were negative. The low overall correlation for the entire sample set indicates that the quantitative relation of serum to salivary estradiol varies substantially between individuals.

Among women in the Conception Study, the mean salivary estradiol plotted across the non-conception menstrual cycles for 11 women indicates a clear ovulatory peak (Figure 1). For conception cycles, a significant rise in estradiol could be detected as early as the 11<sup>th</sup> day of the luteal phase (Figure 2). For salivary progesterone, a single luteal peak similar to that observed in serum was found in the non-conception cycles (Figure 3), and a significant elevation of progesterone could be detected as early as luteal day 9 (Figure 4).

As stated above, we have completed measurements for estradiol and progesterone in all samples from TCPS participants. Our chief goal was to evaluate the 'tracking' of estradiol and progesterone levels from one cycle to the next, within subjects. Progesterone profiles for consecutive cycles from the same woman were often quite similar (see example, Figure 5). Estradiol profiles exhibited a smaller amplitude of variation across the cycle and greater day-to-day variability. Nonetheless, similarities between consecutive cycles were observed (see Figure 6). The intraclass correlation coefficients (ICC) for progesterone are shown in Table 3 below.

Table 3: Cycle-to-cycle variability in peak and cumulative salivary progesterone: The TCP Study

	#cycles	I.C.C.
Peak (by 3-day running mean)	24	0.68
Cumulative (days +2 to +9)		
a. Reverse dating	24	0.72
b. LH dating	20	0.74
c. Rise in progesterone	20	0.77

The results indicate substantially greater variance between compared to within women for both peak and cumulative progesterone. The peak was determined as the maximum 3-day running average. Cumulative progesterone was estimated by adding daily values for days +2 to + 9, using 3 different methods for localizing the mid-cycle (day 0) point. These results substantiate the inference that both peak and cumulative progesterone levels are consistent from one cycle to the next, relative to differences between women.

Although the graphs of individual profiles for salivary estradiol do not appear to be as consistent for individual women as the profiles for progesterone, the intraclass correlation coefficients for cumulative estradiol were actually higher (see Table 4).

Table 4: Cycle-to-cycle variability in cumulative salivary estradiol: The TCP Study

	Intraclass Correlation Coefficient		
	Reverse dating	LH dating	Rise in PG
Pre-ovulatory (days -4 to 0)	0.69	0.72	0.75
Center cycle (days -6 to +8)	0.81	0.90	0.86
Whole cycle (except first 5 days)		0.91	

The high ICC for the center of the cycle (adding days -6 to +8) is undoubtedly due in part to the use of data from 15 consecutive days – but this is the advantage of using saliva for daily measurement. The large day to day variation is partly compensated for by the ability to collect and combine samples from many consecutive days. We note that when we used data for the whole cycle (minus the first 5 days), 91% of the total variance was attributable to between woman differences. This suggests that cumulative or integrated estradiol exposure is highly variable between women – even in an affluent Western population. Other studies we have conducted suggest extreme differences in ovarian hormone levels between Western women and women in non-industrialized cultures.

It will often be too costly and time-consuming to assay each individual daily saliva sample separately in clinical or epidemiologic studies. A far more cost-efficient approach would be to combine aliquots from a number of consecutive days in a single tube and perform a single assay on that tube. We analyzed our data to determine the effects on variance for estimation of mid-luteal estradiol and progesterone when varying numbers of daily samples are combined. The results are shown in Table 5.

Table 5: Cycle-to-Cycle Variability in Mean Mid-Luteal Salivary Progesterone and Estradiol: Comparing Different Methods for Locating Mid-Luteal Phase and Varying Number of Samples Used to Calculate Mean.

	Intraclass Correlation Coefficients			
	Number of days used to estimate mean			
	1	3	5	7
<u>Progesterone</u>				
Reverse dating	0.43	0.69	0.70	0.71
LH dating	0.76	0.84	0.73	0.76
Rise in PG	0.41	0.65	0.78	0.82
<u>Estradiol</u>				
Reverse dating	0.45	0.60	0.79	0.87
LH dating	0.58	0.74	0.84	0.94
Rise in PG	0.52	0.60	0.69	0.74

As expected, these results indicate that the within woman variance is reduced as one increases the number of samples considered. However, the difference between using 5 versus 7 samples is not very great. We also note that for progesterone ICCs with 5 and 7 days are highest with rise in PG as the method for locating cycle position, whereas the ICCs with reverse dating are the lowest. This is as predicted, because reverse dating assumes an equal luteal phase length for each cycle. For estradiol, LH dating appears to be best, followed by reverse dating.

#### **A. Methods and Procedures: Project 2 (Lobular Differentiation in Normal Breast Tissue)**

##### **Case-control study**

We have completed data collection for a case-control study on the relation of lobular differentiation to the risk of breast cancer. This study encompasses, and extends beyond Task 3 in the original SOW. The specific aims of this study are to apply the index to conduct pilot studies exploring the relationship between lobular differentiation and a.) breast cancer risk, and b.) reproductive and hormonal variables hypothesized to be determinants of breast tissue maturation.

Thus far, we have randomly selected 32 patients under age 55 diagnosed with invasive or in-situ breast cancer since 1995 at Northwestern Memorial Hospital. We also randomly selected 30 patients who had recent biopsies that did not reveal malignant disease. Age was restricted to younger women to reduce distortion of lobular typing by age-related lobular regression. Slides from each patient were scored for lobule type by validated readers. Previous results indicate that the number of lobules read can be limited to 100. Dr. Wiley initially reviews all cases and all slides containing breast cancer are not sent to the reader to

avoid bias. Analysis is based on the difference in lobule type distribution (types 1, 2 or 3) in cases versus controls. We hypothesize that cases will have a higher percentage of type 1 lobules and a lower percentage of type 3.

## B. Results/Discussion: Project 2

We identified 4 patients in our breast cancer series (2 infiltrating, 2 DCIS) who were diagnosed with breast cancer within 2 years postpartum. Because pregnancy is hypothesized to produce a temporary shift towards a more mature lobular pattern, we examined these cases separately. In fact, the mean % type 1 lobule among these cases was quite low compared to all controls - 61.4% - and the % type 2 was elevated - 26.5%. One woman who was diagnosed during pregnancy had the highest % type 3 we observed - 36%. Although these data are sparse, they lead us to conclude that recent childbirth (or at least progression to late pregnancy) is associated with a differentiated lobular pattern. These 4 cases are therefore excluded from further analysis to reduce bias. We also identified one case with a predominant lesion of fibroadenoma and a small focus of lobular neoplasia (also termed LCIS). We elected to exclude this case also because the distinction between lobular neoplasia and severe atypical hyperplasia - which is represented in the "benign" group - is very small.

Table 6 shows the comparison of lobule type distribution in breast cancer cases versus benign controls. Considering all subjects, breast cancer cases (including DCIS) had a slightly higher percentage of type 1 lobules and slightly lower percentage of type 2, but the difference is easily consistent with chance ( $P = 0.36$  for type 1). Six patients in the benign group were under age 30 - including 4 with a diagnosis of fibroadenoma, 1 with stromal fibrosis and 1 breast reduction. These subjects had markedly higher percentages of immature lobules. When we restrict analysis to subjects 30 years of age or older, the age of cases and controls becomes similar. In this subgroup, the mean type 1 % for cases is 7.3% higher than the type 1 % for controls, and the type 2 % is 5.7 % lower. These differences do not reach conventional statistical significance levels, however. We also stratified subjects by parity status. All parous subjects were older than 30. In this stratum, breast cancer cases had a type 1 % 10.6% higher and type 2 % 9.8% lower than controls. These results were also of borderline statistical significance.

Table 6. Distribution of lobule types in breast cancer cases versus controls with benign breast histology (all P values are two-sided)

	N	Type 1,%	Type 2,%	Type 3,%	P value, Type 1	P value, Type 2
All subjects						
breast cancer	27	82.8	12.6	4.6		
benign	30	78.9	16.0	5.5	0.36	0.24
Subjects $\geq$ age 30						
breast cancer	27	82.8	12.6	4.6		
benign	24	75.5	18.3	6.2	0.08	0.05
Parous, $\geq$ age 30						
breast cancer	16	83.2	12.4	4.4		
benign	12	72.6	21.2	6.0	0.09	0.05

Altogether, the results of this pilot study suggest a possible relation between the histologic lobule differentiation marker and breast cancer, but the data are far from



conclusive. The greatest difference between cases and controls was observed among parous women – which is consistent with the hypothesis that some women have a sub-optimal differentiation response to pregnancy and thus acquire a persistently elevated risk of breast cancer. A larger study would provide more definitive answers. In addition, a larger study would allow for additional control over factors such as parity, age, lactation and time since last pregnancy. Our data do indicate that during a relatively short interval after childbirth, lobular differentiation shifts to a more mature pattern. Future studies should include larger numbers of older women – despite the tendency for lobule type to regress with aging – because a substantial proportion of breast cancers in younger women is related to inherited factors that might not impinge on differentiation.

We plan to complete analysis of these data shortly, and then decide about preparing a manuscript. We have begun discussions with colleagues who have access to a large set of archived biopsy sections from women who subsequently did or did not develop breast cancer. These materials would be ideal for a nested case-control study of our differentiation marker. However, the labor involved (an average of 1-1.5 hours per subject) in screening and reading slides and the softness of the results from our pilot study advise caution. We plan to discuss our results as widely as possible in order to solicit suggestions as to how and whether the histologic marker can be improved.

## CONCLUSIONS

During the previous project year, significant accomplishments of this project include:

- Completion of development of reliable, valid assays for salivary progesterone and estradiol. The estradiol assay is the first direct (non-extraction) assay to be developed, and our reliability/validation data are extensive. A manuscript describing these results is currently under a second round of review at a peer-reviewed journal.
- Completion of data collection and analysis of studies using salivary estradiol and progesterone to estimate the cycle-to-cycle variability of integrated hormone levels in premenopausal women. These studies, because they avoid daily venipuncture, are among the the largest and most complete to date on cycle-to-cycle variability in ovarian steroid level, and are being prepared for publication.
- Completion of several steps in the development of the assay for estradiol in breast fluid. The effects of various extraction procedures on background and recovery have now been clarified.
- Acceptance of a manuscript on differences in TGF- $\alpha$ , EGF and TGF- $\beta$  in expressed prostatic fluid from men with prostate cancer, benign hyperplasia and normal prostates. This work relates closely to the assay development work we are undertaking with breast fluid, and is currently being used to improve the TGF- $\beta$  assay for breast fluid.
- Start of assays for TGF- $\alpha$  in breast fluid samples from the Mammographic Density Study. When completed, these data will complement existing data relating EGF levels in breast fluid to breast tissue density as measured from mammograms.

- Completion of our pilot study on the relation of breast lobular differentiation pattern to breast cancer development. Using the histologic classification method previously developed under this project, we compared lobule types in normal breast tissue from 32 women with breast cancer and 30 women with benign breast histology. We found weak associations between nulliparity and less developed lobular patterns, and have some data corroborating the effect of recent completed pregnancy on lobular differentiation. Our data also provide suggestive evidence that an association between immature lobule type and breast cancer might exist among parous women.

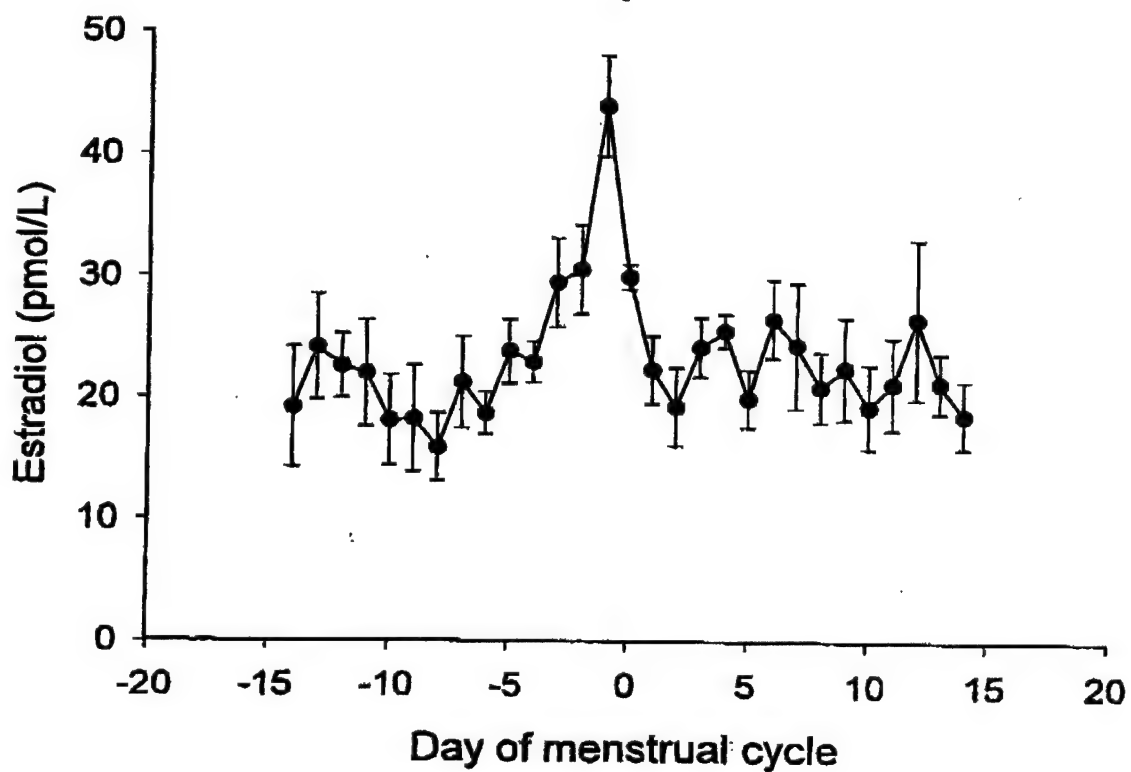


Figure 1. Nonconception cycles. Levels of estradiol in daily saliva samples from 11 subjects who were planning a pregnancy (means  $\pm$  SE). Day zero is the day after the midcycle peak of salivary estradiol concentrations.

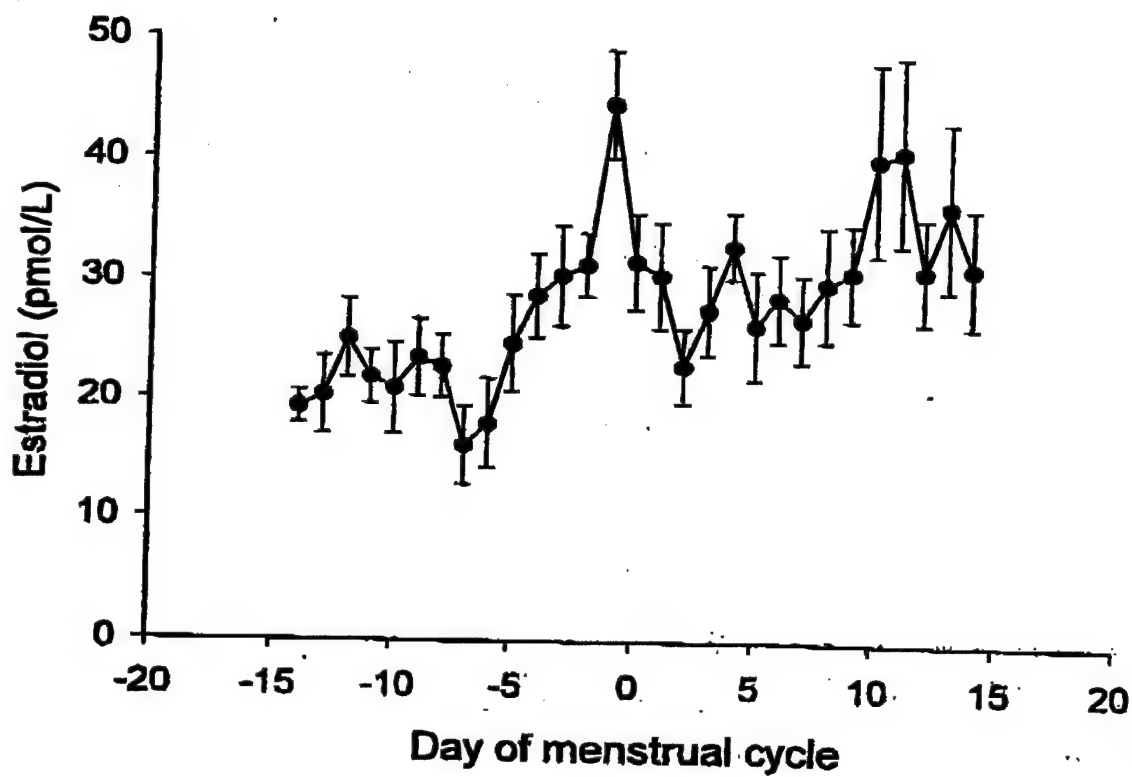


Figure 2. Conception cycles. Levels of estradiol in daily saliva samples from 11 subjects who were planning a pregnancy (means  $\pm$  SE). Day zero is the day of the first decline after the midcycle peak of salivary estradiol concentrations.

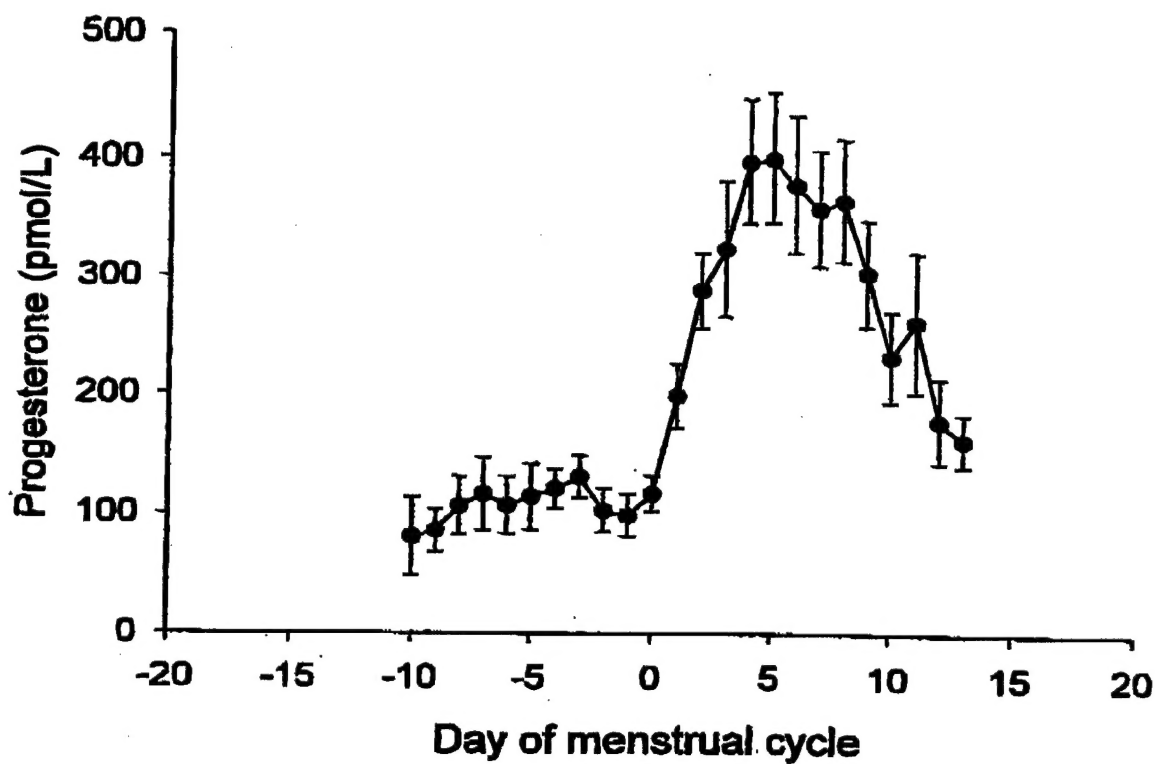
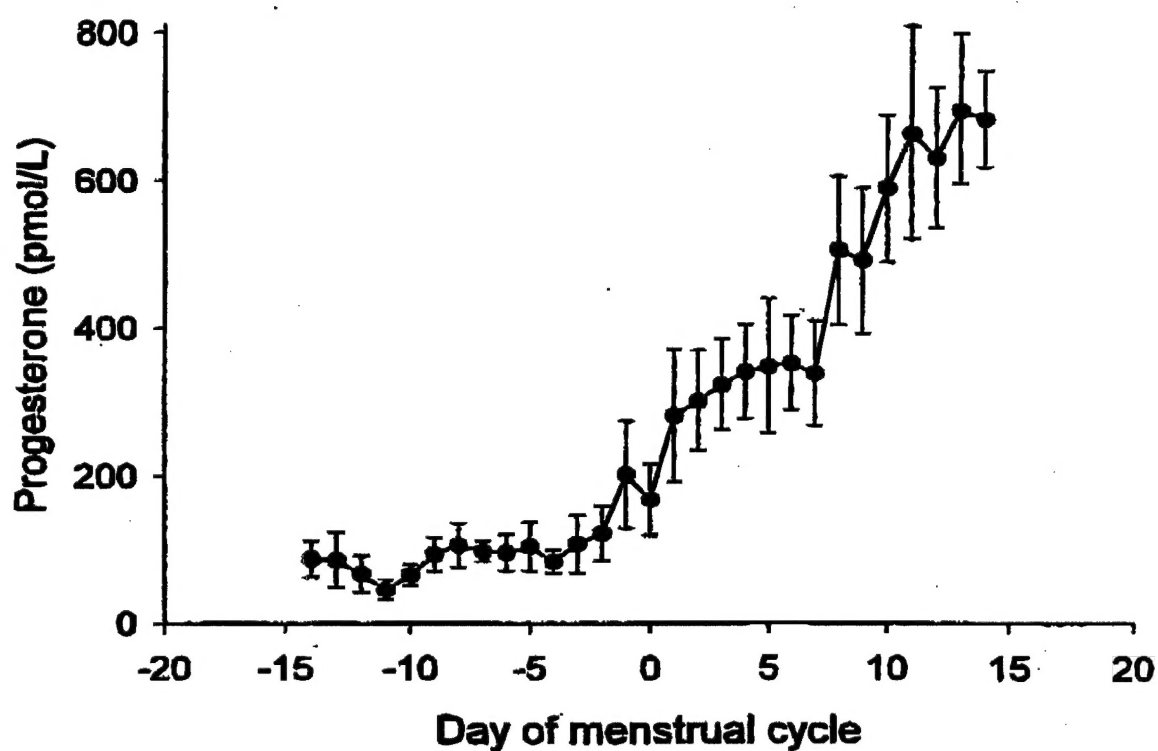
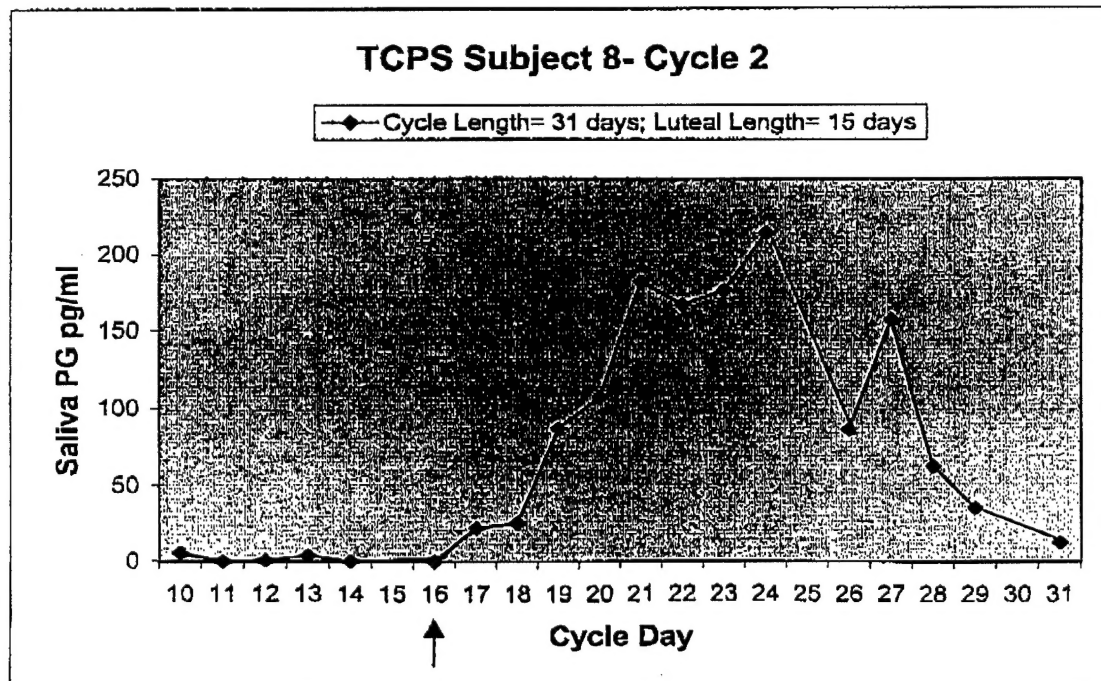
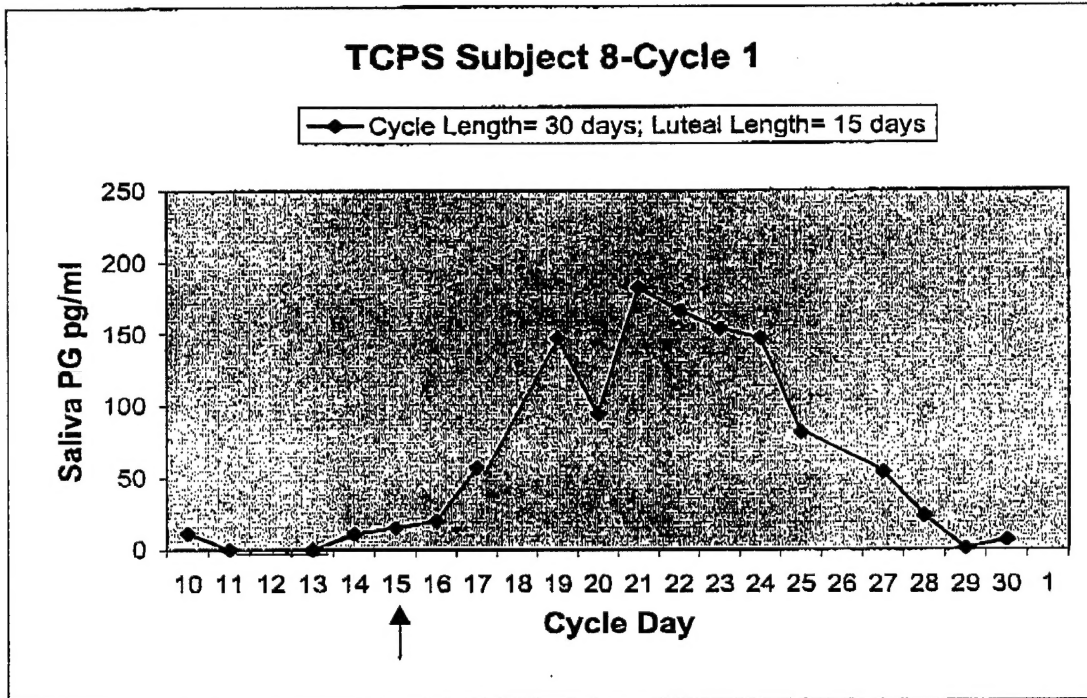


Figure 3. Nonconception cycles. Levels of progesterone in daily saliva samples from 11 subjects who were planning a pregnancy (means  $\pm$  SE). Day zero is the day of the first decline after the midcycle peak of salivary estradiol concentrations.



**Figure 4. Conception cycles. Levels of progesterone in daily saliva samples from 11 subjects who were planning a pregnancy (means  $\pm$  SE). Day zero is the day of the first decline after the midcycle peak of salivary estradiol concentrations.**

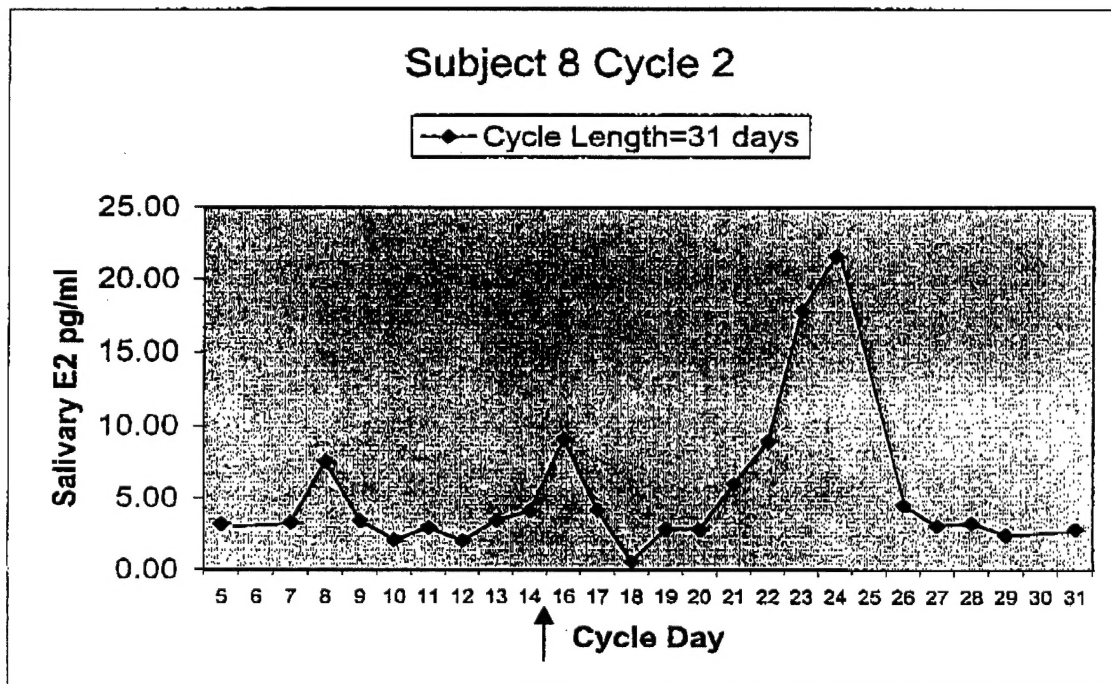
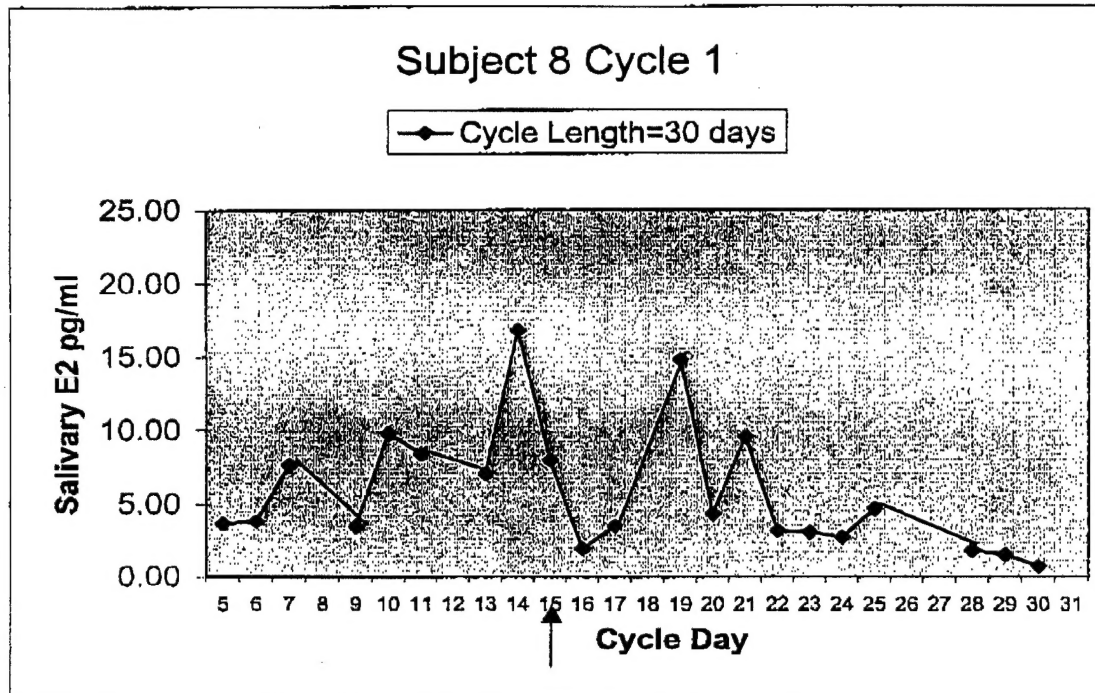
Figure 5 Progesterone



TCPS Saliva Progesterone  
Arrows indicate LH peak



Figure 6 Estradiol



\*\* Cycle 1 Day 14 sample was pink, so may be contaminated